IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Beer et al.	Examiner: A. Kubelik
Serial No.	:	09/770,693	Art Unit: 1638
Cnfrm. No.	:	6816	
Filed	:	January 26, 2001	
For	:	OOMYCETE-RESISTANT TRANSGENIC PLANTS BY VIRTUE OF PATHOGEN-INDUCED EXPRESSION OF A HETEROLOGOUS HYPERSENSITIVE RESPONSE ELICITOR	

DECLARATION OF STEVEN V. BEER AND DAVID W. BAUER UNDER 37 CFR § 1.131

We, Steven V. Beer and David W. Bauer, hereby declare:

1. We are the inventors of the above-identified application.

2. We are presenting this declaration to demonstrate that the claimed subject matter was invented, in the United States, prior to February 1999. As demonstrated below, the claimed invention was reduced to practice prior to February 1999.

3. Attached hereto as Exhibit A is a copy of a disclosure document that we submitted to Cornell Research Foundation as part of an invention disclosure form. The disclosure document, characterized as a brief summary, is entitled: "Disease Resistant Transgenic Plants by Virtue of the *hrpN* Gene of *Erwinia amylovora*." This copy of the disclosure document is a what was received by Cornell Research Foundation following facsimile transmission by us, specifically by Steven V. Beer. That this document was sent via facsimile transmission is evidenced by the header imprinted on top of the pages. The phone number 607-255-4471 is the number dedicated to the fax machine in the Cornell University Department of Plant Pathology. Although the date of the document has been redacted, the date of the disclosure document is prior to February 1999. The date imprinted in the header has also been redacted but it, too, is prior to February 1999.

4. The disclosure document attached as Exhibit A describes the formation of a chimeric gene that includes the potato *prp1*-1 promoter, *hrpN* coding sequence (from *Erwinia amylovora*), and the 3' transcription terminator of the nopaline synthesis gene of *Agrobacterium tumefaciens*. In addition, an alternative embodiment of the chimeric gene

included the signal sequence of tobacco PR1b, which was cloned between the promoter and the *hrpN* coding sequence.

5. The different chimeric genes were introduced into disarmed *Agrobacterium tumefaciens* strain GV3101 and then, using standard Arabidopsis vacuum infiltration transformation procedures, transformed into wild-type Arabidopsis ecotype Col-0. The transformed Arabidopsis plants were allowed to self-fertilize and progeny homozygous for the *hrpN* construct were selected. Three different lines transformed with the *hrpN* gene under control of the prp1-1 promoter, with and without the signal sequence, were selected for testing.

6. Plants were inoculated with *Peronospora parasitica* strain Noco, an oomycete, and then assessed for disease on a daily basis for three weeks. All control plants, including wild-type Col-0 and null transformants, developed sporulating lesions by Day 9 post-inoculation. Two of the *hrpN*-containing plants developed sporulating lesions by Day 14 and one only showed sporulating lesions beginning on Day 16. These results demonstrate that the *hrpN* coding sequence under control of the *prp1*-1 promoter can be effective to provide partial resistance against oomycete infiltration.

7. In addition to the above results, the document attached as Exhibit A also indicates that the same constructs were being used to produce transgenic apple, tomatoes, and potatoes with the assistance of other Cornell University researchers specializing in those particular plants. The document also indicates that apple trees will be evaluated for resistance to the oomycete *Phytophthora cactorum* and tomato and potato plants will be evaluated for resistance to the oomycete *Phytophthora infestans* (which causes late blight).

8. With regard to other individuals identified in the document attached as Exhibit A, particularly Eric R. Garr, Herb Aldwinkle, John Norelli, Steven Tanksley (Dr. Tanksley), and Steven Slack (Dr. Slack), none of these individuals contributed to the conception of the invention defined in the present application. The invention was conceived by us. In making our invention, experiments were conducted by us or under the direction and control of one or both of us.

9. Eric Garr was a graduate student working in the Beer laboratory, under both of our direction. Eric was involved in preparing the chimeric gene and carrying out assays in *Arabidopsis* to determine efficacy of the chimeric gene in the transformed plants, as

described in paragraphs 4-6 above. Eric performed these tasks under the direct supervision of David Bauer.

10. Drs. Aldwinckle, Norelli, Tanksley, and Slack are other researchers who were at the time at Cornell University. Each of these researchers has expertise in different plants. After having conducted the work in *Arabidopsis*, described in paragraphs 4-6 above, we provided a chimeric gene of the present invention to these other researchers and asked them to test the chimeric gene for efficacy in their plants of expertise. The experiments they performed were done so exclusively at our request.

11. We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: April 23, 2004

Steven V. Beer

Date: April ___, 2004

David W. Bauer

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Date: April ___, 2004

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Steven V. Beer

Date: April 29, 2004

David W. Bauer
David W. Bauer

Disease Resistant Transgenic Plants by Virtue of the *hrpN* Gene of *Erwinia amylovora*

BRIEF SUMMARY

David W. Bauer, Eric R. Garr and Steven V. Beer
Department of Plant Pathology, Cornell University, Ithaca, NY 14853
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Recent research results have indicated that the *hrpN* gene of *Erwinia amylovora* confers resistance to plants normally susceptible to a disease caused by an oomyceteous fungus. These results culminate several years of work involving molecular genetics of bacterial and plant systems. They are summarized briefly below.

In principle, we sought to confer resistance to plants by expressing the gene that encodes harpin, a protein that elicits the hypersensitive response (HR) when sufficient quantities are infiltrated into the intercellular spaces of leaves of most plants. The HR is a resistance reaction by plants toward potential pathogens where the plant cells rapidly die and the pathogen can no longer grow. When applied externally to plants, harpin also induces systemic acquired resistance (SAR), growth enhancement, and insect repellency. To prevent the development of HR, which is lethal to the plant tissues involved, the *hrpN* gene must be controlled by a promoter that provides expression of *hrpN* only when a plant part is threatened by a pathogen. We selected a promoter from potato, the *prp1*-1 gene, which is activated by the oomyceteous fungus *Phytophthora infestans*, and not by wounding or other environmental perturbations, which often activate other pathogen-inducible promotors (described in U.S. Patent No.5,750,874, which was assigned to the Max-Planck Gesellschaft Zur Forderung in Munich, Germany).

Phytophthora infestans, an oomycete, causes the dread disease known as late blight. The disease was responsible for the Irish potato famine, and has become very important recently due to the development of a highly virulent new strain that is capable of causing severe disease losses in tomatoes as well as potatoes. In addition, the new isolate is resistant to the fungicides that had been used successfully for control until now. Present means of control are limited; some very new and expensive fungicides provide some control of the new strain.

The promoter from the *prp1*-1 gene was isolated by PCR amplification of the region from genomic DNA of the potato variety Atlantic. The *hrpN* gene of *Erwinia amylovora* was cloned under the control of the *prp1*-1 promoter in plant transformation vectors pPZP211, pPZP221 and pBINPLUS (none known to be patented). Separate constructs were made that included a signal sequence needed for secretion of proteins from plant cells. The signal sequence, PCR amplified from the *PR1b* gene of tobacco, was cloned between the promoter and the *hrpN* gene. In each construct, a

transcription terminator, derived from the nopoline synthesis gene of *Agrobacterium tumifaciens*, was included to provide the poly A tail for expression in plant cells. The constructs described above were introduced into disarmed *Agrobacterium tumefasciens* strain GV3101 for use in transforming *Arabidopsis thaliana* plants. The constructs were transformed into wild-type Arabidopsis ecotype Col-0 using standard Arabidopsis vacuum infiltration transformation procedures.

Three different lines transformed with the *hrpN* gene under control of the *prp1*-1 promoter, which previously had been shown to be homozygous for the *hrpN* construct, with and without the signal sequence, were selected. These were inoculated with *Peronospora parasitica* strain Noco, an oomycete that causes downy mildew disease of Arabidopsis. The plants were inoculated by placing drops of an aqueous suspension of freshly harvested sporangia of the pathogen on the leaf surfaces. The inoculated plants were incubated under conditions conducive to downy mildew development and disease assessments were made daily for three weeks. All of the control plants, including untransformed ecotype Col-0 and Col-0 transformed with pBI101 or pBI121, developed sporulating lesions by Day 9 after inoculation. Two of the *hrpN* containing lines, 6-1 and 9-1, developed sporulating lesions by Day 14 after inoculation. One *hrpN* containing line, 6-2, did not develop sporulating lesions until Day 16 after inoculation. These results show that *hrpN* under control of the *prp1*-1 promoter delayed the development of disease and provided a measurable amount, but not complete, resistance to downy mildew.

These results are important because useful resistance often is due to a delay in disease development rather than complete immunity. Also, we postulate that the constructs containing the signal sequence fused to *hrpN*, for secretion from the plant cells, will result in even greater levels of resistance. This is because previous experiments have indicated that harpin acts from outside the plant cells to elicit the HR. We speculate that the resistance in the plants described above is due to leakage of harpin out of the plant cells which then induces the HR and resistance. The process should be much more efficient with the signal sequence.

The transgenic lines described above are currently being retested. In addition, other lines containing the *hrpN* gene without the signal sequence are under test. We now have transformants with *hrpN* and the signal sequence under control of the *prp1*-1 promoter. The lines will be tested for resistance to downy mildew as soon as the seeds are available. In addition to downy mildew, we are also testing our transgenic Arabidopsis for resistance to powdery mildew, caused by *Erysiphe cichoracearum*, and bacterial blight caused by *Pseudomonas syringae* pathovar tomato. Neither of these pathogens are oomycetes and it is not clear that the *prp1*-1 promoter will respond to pathogens other than oomycetes.

The same constructs as used with Arabidopsis are being tested in apple by Drs. Herb Aldwinckle and John Norelli of Cornell's Geneva campus. Their programs and labs emphasize apple improvement through breeding and genetic engineering. The joint project aims at developing apples with greater resistance to fire blight. Apple rootstocks and scions have been/are being transformed with the constructs. The resulting transgenics will be evaluated for resistance to the important apple diseases caused by *Erwinia amylovora*, *Venturia inaequalis*, and *Phytophthora cactorum*. The production and evaluation of transgenic apple requires much more time than similar operations with herbaceous plants. Thus, the apple work was begun as soon as possible, essentially at the same time that the work with Arabidopsis was begun. Preliminary disease evaluations of transgenic apple are anticipated in approximately 6 months, assuming our future progress continues at the same pace as previously.

Based on our results with Arabidopsis, we initiated projects with other Cornell scientists (Drs. Tanksley and Slack) to transform tomatoes and potatoes with our constructs and test the resulting transgenics for resistance to late blight. Even if the *hrpN*-containing constructs provide only partial resistance to oomycetes, they still will be very valuable. Late blight of potatoes and tomatoes are two of the most important diseases in the world, economically, and growers spend huge amounts of money on fungicides to combat them. In addition, some downy mildew diseases of other plants (grape, tobacco and lettuce, for example) also are highly important economically.